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NEGATIVE AND POSITIVE FEEDBACK CONTROL OF THE COMMITTED GRANULO--ETC(U)
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EXPERIMENTAL HEMATOLOGY TODAY

Edited by
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INTRODUCTION

In general the hematopoietic system is comprised of three main compartments: (1) the pluripotential stem cell; (2) the committed stem cell; and (3) the differentiated compartment (Fig. 1). The pluripotent stem cells as represented by the colony-forming unit-spleen (CFU-s) have the physiologic capabilities of self-renewal and control of compartment size, which are normal requirements of precursor cells (30). Regulation of this compartment is assumed to be, at least in part, by short-range, inhibitory, cell-to-cell interaction, and it is possible that modification of cell membranes may affect recognition and initiate increases in compartment size (23). In addition, there exists evidence that the CFU-s are also influenced by long-range humoral agents (2, 20, 32). However, at this time we have no precise knowledge of the conditions responsible for the initiation of the differentiation of CFU-s into committed stem cells, such as the CFU-c. It has been hypothesized that the known migratory behavior of CFU-s, which carries them into the circulation and from there to sites of hematologic significance, eventually places them within a favorable hematopoietic microenvironment for their conversion into CFU-c. It is plausible that long-range stimulatory factors, which may or may not be identical with the colony-stimulating activities (CSA), initiate the migration of CFU-s. How and by what means they eventually select the suitable ecologic niches is an enigma at present.

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Negative and Positive Feedback Control of the Committed Granulocytic Stem Cell Compartment

Siegmund J. Baum

1977

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PHYSIOLOGIC CONTROL OF THE CFU-c

The topic of the present discussion is the physiologic control of the CFU-c which have arrived in a favorable microenvironment. As indicated schematically in Fig. 1, the cells in the CFU-c compartment are not self-sustaining. As they proliferate and differentiate into granulocytes and monocytes, their number must be replenished by the CFU-s compartment. Normally, this is achieved by the release of a small number of CFU-s, and the majority of these cells remain in the resting or G_0 state. If in response to injury the CFU-c compartment becomes depleted, CFU-s go into cyle and a larger number of cells are released. The CFU-s as well as the CFU-c must achieve a critical size before cells are released into the next compartment. Once the cells have become CFU-c there must be amplification and differentiation prior to efflux into the differentiated compart-

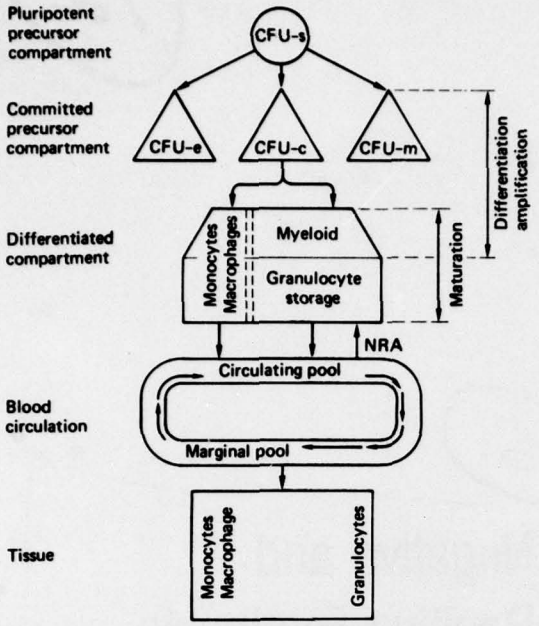
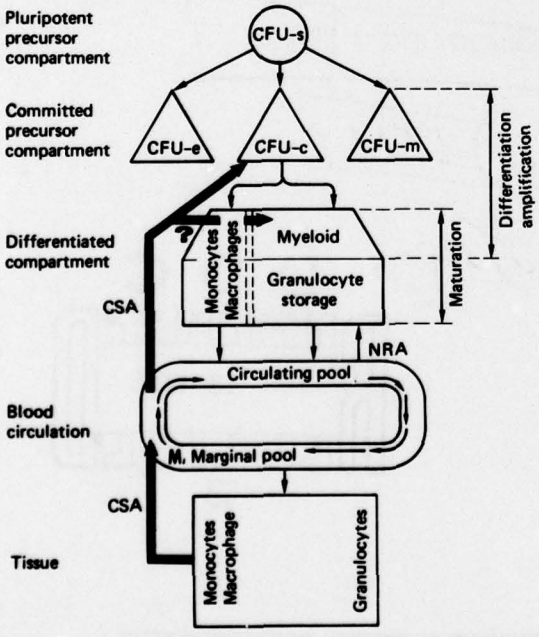


FIGURE 1. Schematic model of normal leukopoiesis.

ment. The CFU-c differentiate into recognizable cells, the myeloblasts; further amplification and differentiation occurs until the cells are stored in the bone marrow as metamyelocytes, bands, and mature polymorphonuclear leukocytes. This bone marrow storage pool contains about 4–6 times the number of cells present in the circulating blood. Cells leave the marrow storage pool in a sequential manner, the older cells being released first (13). Recent findings indicate that aging of granulocytes is associated with deformability and decreased stickiness (17). This obligatory maturation would, of course, favor a sequential release from the storage pools.

Upon stimulation by antigens such as endotoxin, a great number of the stored cells are released into the periphery. As may be seen from Fig. 1, a protein originally discovered by Gordon and his associates (14) may be responsible for this leukocytosis by causing a discharge of granulocytes from the storage pool. This factor was originally called leukocytosis-inducing factor; recently, however, it was more appropriately renamed neutrophil-releasing activity, or NRA (31). The transit time of the released leukocytes in the circulation is relatively short—a matter of several hr—and those cells which do not remain in the marginal pool migrate to the tissues and become the primary defense against invading antigens.

FIGURE 2. Schematic model of the positive feedback system for leukopoiesis.



POSITIVE FEEDBACK SYSTEM FOR CFU-c REGULATION

As may be seen in Fig. 2, all the leukocytic compartments discussed above have been implicated from time to time as possible sources of stimulatory factors or leukopoietins involved with the entry, amplification, differentiation, and release of the CFU-c. The development of an assay which permitted the cloning of marrow cells, or more specifically CFU-c, in a semisolid medium (4, 25) finally demonstrated the existence of a specific stimulating factor (22). This factor was originally named colony-stimulating factor (CSF), and more recently was renamed colony-stimulating activity (CSA) (16). For the present discussion we are making the assumption that CSA is identical or related to the humoral factor responsible for *in vivo* CFU-c turnover. However, it is understood that while this concept is accepted by some workers (26), it is questioned by others (24). The prime candidate for the origin of CSA is the monocyte/macrophage system (31). In our own laboratory, we have demonstrated that the 24 hr cellular concentration in a model of an inflammatory exudate, which consisted of an acrylic cup filled with

Hanks's balanced salt solution and placed in a subcutaneous pouch in rodents, contained primarily monocytes (34). Cells or supernatant removed from this cup, or plasma from mice with implanted cups, stimulated increased formation of CFU-c on agar plates, as seen in Table 1. However, they also stimulated increased CFU-s formation (2).

In summary, Fig. 2 represents a conceptional model of the stimulatory feedback system for the CFU-c compartment. Presumable humoral substances produced by monocytic cells in inflammatory tissues stimulate CFU-c into cell cycle. It is quite possible that the same substances also initiate CFU-s turnover and the release of these cells into the CFU-c compartment. The relationship of CSA to NRA is not understood, at present. However, evidence was presented for a separate identity of the two (31). Although increased NRA and CSA are produced in response to endotoxin administration, the former, which mobilizes granulocytes from the storage pool, may be produced by leukocytes in the blood, whereas the latter is released by monocytes/macrophages in the tissues in response to endotoxin, and stimulates CFU-c into cycle. It is doubtful that CSA is produced in the circulation. Table 2 summarizes the known physical properties of CSA.

TABLE 1 Number of CFU-c per 10^5 Bone Marrow Cells in Mice

INJECTION	MEAN NUMBER OF COLONIES PER PLATE
Exudate plasma[a]	149 ± 33.6[c]
Exudate supernatant	141 ± 36.0
Exudate cells	115 ± 16.8
Hanks's solution	102 ± 16.2
Normal plasma[b]	100 ± 12.7

[a]Plasma from mice with implanted acrylic cup.

[b]Acrylic cup not implanted.

[c]Standard error.

NEGATIVE FEEDBACK SYSTEM FOR CFU-c CONTROL

Although one could easily accept a model of CFU-c control under the influences of CSA alone, evidence from other studies clearly indicates a more complex physiologic system. Craddock et al. (11) demonstrated that effective withdrawal of large numbers of granulocytes from the blood leads to an accelerated release of cells from the bone marrow in dogs. In contrast, infusion of autologous mature granulocytes into the circulation of dogs reduced cell release from the marrow, and consequently inhibited granulocyte production (12). Destruction of leukemic cells by extracorporeal radiation of the blood appears to stimulate proliferative activity of leukemic blast cells in the bone marrow (7). Transfusion of fresh blood apparently causes a transient fall in the white blood cells of leukemic patients (35). Several of these authors suggested the operation of a negative feedback system under the control of inhibitory agents or chalones (6, 10, 27).

In earlier studies designed to determine the existence and biologic specificity of the granulocyte chalone in *in vitro* bone marrow cultures, radioautograph labeling with ^{3}H-thymidine (^{3}H-Tdr) showed that extract of granulocytes significantly decreased granulocytic precursors (28). No changes in labeling index were detected for other bone marrow cells. Although some doubts were expressed about the validity of these findings (27), recently, using a more refined technique of analysis, the strict specificity of action of partially purified extracts from granulocytes on their own precursors was confirmed (1). The effects of extracts from mature granulocytes can be determined by measuring the structuredness of the cytoplasmic matrix of the receiving cells (19). Such studies have clearly demonstrated that granulocyte extracts affect only granulocytic precursor cells. Incidentally, these fractions were tested at concentrations of *33 μg/ml*.

Studies utilizing diffusion chambers in an *in vivo* closed culture system have given a better understanding of the specific action of granulocytic chalone. In an early experiment, extracts of granulocytes and of liver were injected into mice implanted with two different culture chambers (3). It was shown that granulocyte extracts inhibited DNA synthesis in proliferating granulocytes

TABLE 2 Characterization of CSA

Chemical composition	Glycoprotein
Temperature of inactivation	Above 60°C
Sedimentation coefficient	4.5–7.0
Electrophoretical migration	α-Globulin-post albumin range
Molecular weight by gel filtration	70,000
Sucrose gradients	45,000
Human CSA by gel filtration	45,000
Inactivated by	α-Chymotrypsin, subtilisin

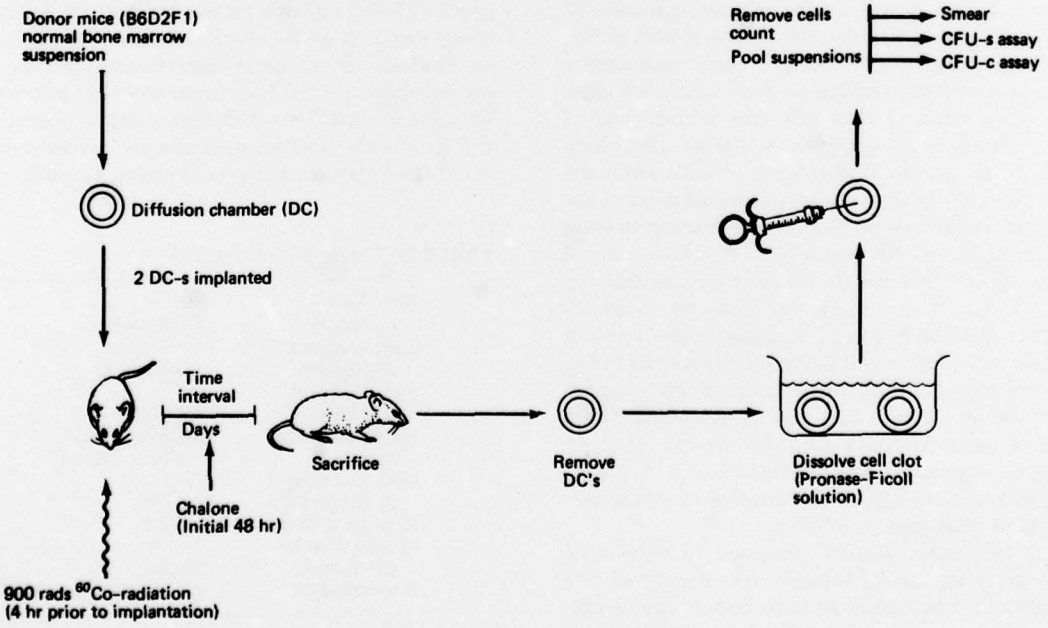
but not in immunoblasts and macrophages. It appears, then, that the granulocyte chalone, like other chalones, is a cell line-specific but not species-specific regulator substance. It inhibits cell proliferation in the granulocyte system in a reversible manner. The latter was established in an interesting experiment by Laerum and Maurer (15).

In our own laboratory, Drs. MacVittie and McCarthy (21) used the *in vivo* diffusion chamber technique for mouse marrow culture to determine the proliferative responses of CFU-s and CFU-c after exposure to granulocyte inhibitor. Presented in Fig. 3 is their experimental design. They prepared a granulocyte-conditioned media (GCM) from granulocytes obtained from rat peritoneal cavities. These cells were induced to accumulate in the peritoneal cavity by injection of 10 ml of 2.5% sterile oyster glycogen. The GCM was derived from media in which white cells were incubated for 20 hr. Based on previous tests by the authors this material contained inhibitors or chalones for granulocytes. Mice (Swiss-Webster) were then radiated with 900 rad ^{60}Co γ-radiation at 154 rad/min. Four hr later they were implanted with diffusion chambers made from 0.22 μm Millipore filters. Each chamber contained separated cell marrow suspensions which were prepared from the femurs of several normal mice in tissue culture media CMRL 10 with 10% fetal calf serum. Two diffusion chambers were implanted into each mouse. The GCM was administered during the initial 48 hr of chamber culture. The diffusion chambers were removed at selected intervals and cells were harvested. Nucleated cell counts were performed prior to pooling the cell suspensions for assay of CFU-s and CFU-c. As is seen in Fig. 4, GCM administration effectively reduced the total nucleated cell production within the diffusion chambers. The specificity of the GCM is attested to by the fact that inhibition of growth did not occur in cultures of mouse fibroblasts (L-929 cells) grown in identical conditions as the marrow cells.

In Fig. 5 it was shown that the GCM containing the chalone has no effect on CFU-s. On the other hand, as can be seen in Fig. 6, the GCM significantly reduced the number of granulocyte progeny formed within the diffusion chamber, in part by reducing cell turnover at the level of the committed granulocyte progenitor cell (CFU-c).

To date it has been difficult to demonstrate chalone activity *in vivo*. However, Schütt and Langen (29) inhibited granulopoiesis in rats by injections of granulocyte extracts. These authors labelled bone marrow cells with ^{3}H-Tdr, and then produced an aseptic inflammation in the peritoneal cavity of rats. The chalone reduced the

FIGURE 3. Flow diagram of the *in vivo* diffusion chamber technique for mouse bone marrow culture.



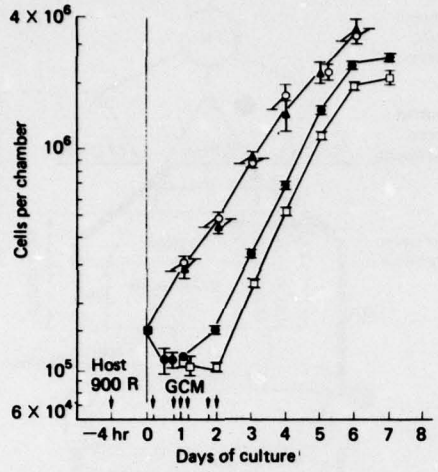
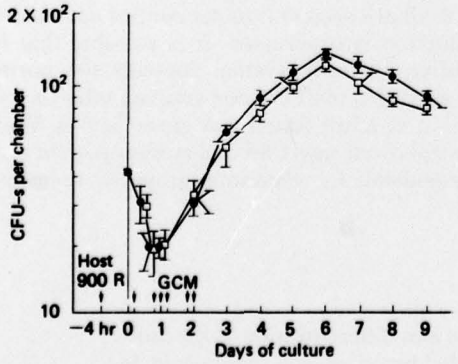


FIGURE 4. The effect of granulocyte-conditioned media (GCM) on the growth of mouse fibroblasts (L-929) inoculated at 10^5 cells per chamber and on nucleated cells (granulocytes and macrophages) inoculated at concentrations of 1.5×10^5 normal bone marrow cells per chamber. Mean values (± SEM) are results of six replicate experiments, and observed differences are significant at $p<0.001$ level. Fibroblasts: ▲, control; ○, GCM. Bone marrow: ●, control; □, GCM. (By permission of the author.)

DNA-specific activity of the granulocytes recovered from the inflammatory exudate by 50%.

All the reports on granulocyte chalone indicate that it inhibits granulopoiesis in a tissue-specific manner. Most likely, cells are arrested in the G_1 phase (15). These studies support the contention that granulocyte chalone is a polypeptide

FIGURE 5. The effect of GCM on the growth of CFU-s in diffusion chambers. ●, Control; □, GCM. Chambers were inoculated with 1.5×10^5 normal bone marrow cells. Values (± SEM) are results of at least four replicate experiments in which chamber cells were pooled and used to inject eight to 10 assay mice per point. (By permission of the author.)



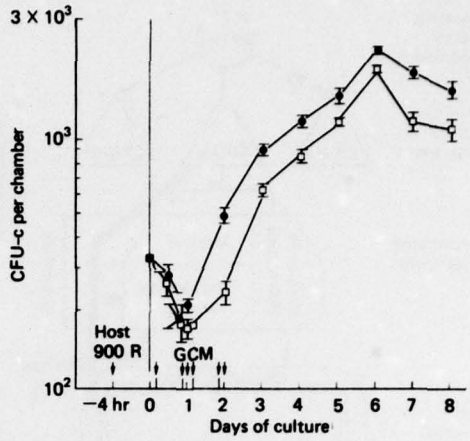


FIGURE 6. The effect of GCM on the growth of CFU-c in diffusion chambers inoculated with 1.5×10^5 normal bone marrow cells. ●, Control; □, GCM. Values (± SEM) are results of at least four replicate experiments in which chamber cells were pooled and used to inoculate four agar plates per point. (By permission of the author.)

with a molecular weight of 4000 daltons (33). However, in addition a nondialyzable inhibitor of granulocytic colony growth has been found in the sera of humans and mice. It is assumed to be a lipoprotein of possibly much higher molecular weight (9).

Figure 7 enables us to summarize the suggested anatomic areas of chalone production and its possible physiologic functions. Nearly all reports seem to agree that granulocytic chalone is produced by adult functional granulocytes (33). These granulocytes were obtained from the tissues (21), the circulation (18), and the bone marrow (26). This, of course, fits precisely the description of a chalone as postulated by Bullough (5). Each cell line or tissue produces specific inhibitors which regulate its mitotic rate. Injury or cell death reduces the concentration of the inhibitor and the inhibition, with a net result of increased mitotic activity and cellular production. Evidence has been presented that the action of granulocyte chalone is on the CFU-c compartment (21).

POSSIBLE INTERACTION OF THE TWO FEEDBACK SYSTEMS IN THE REGULATION OF THE CFU-c COMPARTMENT

A positive feedback system of CFU-c regulation with CSA operating as the controlling agent was described. Evidence was presented that CSA might be produced by monocytes and/or mac-

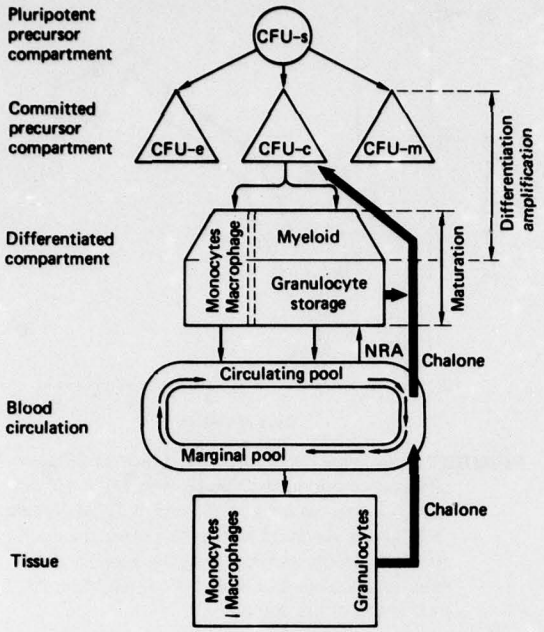


FIGURE 7. Schematic model of the negative feedback system for leukopoiesis.

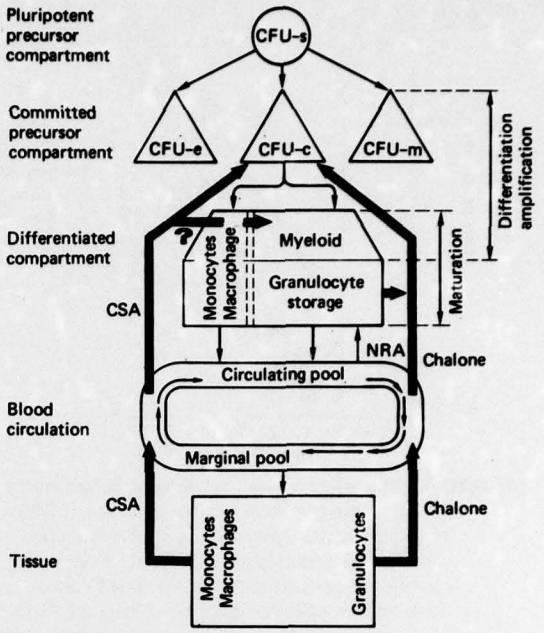


FIGURE 8. Model for leukopoiesis featuring suggested actions of the dual system controlled by both positive and negative feedback systems.

rophages in the tissues, probably in inflammatory loci or exudates. It probably does not act on the bone marrow storage compartment and is not related to NRA, which does release neutrophils from it (31). Although cellular proliferation and differentiation of the CFU-c could easily be accomplished under the stimulatory action of CSA alone, strong evidence has also been produced for the existence of a negative feedback system regulated by the granulocytic chalone (27, 33). The chalone probably is produced by adult functional granulocytes, and its action again might very well be on the CFU-c (21).

Figure 8 presents the possible model for leukocytopoiesis and indicates suggested actions of the dual system controlled by both positive and negative feedback systems. All that remains to be done now is to explain to the satisfaction of the workers in this field how the two regulatory systems may interact. Obviously, if both systems operated simultaneously but independently, responses in times of stress might be chaotic and disastrous. For example, if the chalone titer were high, CFU-c could not rapidly respond to sudden stimulation by CSA. If CFU-c were under strong control of CSA the result would be increased granulocyte production, which in turn would increase the chalone concentration. The net effect could be decreased granulocyte production, regardless of whether or not the stressful condition which initially induced increased CSA production still existed. This situation could obviously not be tolerated. A clue to the possible interaction of the two feedback systems comes from the work of Chan and Metcalf, who reported that wholebody radiation caused an acute, dose-dependent rise in serum CSA levels and concomitant fall in inhibitor level (8). This might well lend support to the idea that under stressful conditions demanding increased granulocyte production, the positive feedback system is under control and chalone production is suppressed. It is possible that the negative feedback system controls the normal concentration of CFU-c and consequently the production of adult functional granulocytes. Under stressful conditions CSA or a granulopoietin alone is responsible for maximum granulocyte production.

SUMMARY

Although there exists some evidence that proliferation and differentiation of the multipotential stem cell (CFU-s) is at least partly controlled by cell-to-cell interaction, it

appears that the committed granulocytic colony forming cell (CFU-c) is under the influence of stimulatory or inhibitory humoral agents. The latter is supported by the fact that the addition of material containing colony stimulating activities (CSA) to the semisolid medium of *in vitro* bone marrow cultures permits the formation of granulocyte and macrophage colonies. CSA has to date only been partially purified and appears to be a glycoprotein with a molecular weight of 45,000 daltons. At present there exists no final proof that CSA stimulates granulopoiesis *in vivo*, however, indirect results from several studies appear to make it a good candidate. Along these lines our studies utilizing either (1) the diffusion chamber methodology and cytoxan treatment of mice or (2) murine models of inflammatory exudates support the hypothesis that CSA represent long distance humoral agents possibly produced by mononuclear cells that migrated into inflammatory exudates.

Shortly after the discovery of CSA, inhibitors of granulocyte-macrophage colony formation were discovered. These inhibitors were separated by gel filtration on Sephadex G-150 into two distinct areas of activity, one being a lipoprotein while the other was not. From diffusion chamber studies it appears that the inhibitors or chalones were specific for CFU-c and have no affect on CFU-s. Furthermore, granulocytic chalones are produced by mature granulocytes and inhibit only granulocytic precursors.

It appears that positive and negative feedback loops regulate CFU-c. CSA as well as granulocyte chalone are candidate regulators of the committed granulocyte colony forming cells. Possibilities of interactions are discussed.

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Although there exists some evidence that proliferation and differentiation of the multipotential stem cell (CFU-s) is at least partly controlled by cell-to-cell interaction, it appears that the committed granulocytic colony forming cell (CFU-c) is under the influence of stimulatory or inhibitory humoral agents. The latter is supported by the fact that the addition of material containing colony stimulating activities (CSA) to the semisolid medium of in vitro bone

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20. ABSTRACT (continued)

marrow cultures permits the formation of granulocyte and macrophage colonies. CSA has to date only been partially purified and appears to be a glycoprotein with a molecular weight of 45,000 daltons. At present there exists no final proof that CSA stimulates granulopoiesis in vivo, however, indirect results from several studies appear to make it a good candidate. Along these lines our studies utilizing either (1) the diffusion chamber methodology and cytoxan treatment of mice or (2) murine models of inflammatory exudates support the hypothesis that CSA represent long distance humoral agents possibly produced by mononuclear cells that migrated into inflammatory exudates.

Shortly after the discovery of CSA, inhibitors of granulocyte-macrophage colony formation were discovered. These inhibitors were separated by gel filtration on Sephadex G-150 into two distinct areas of activity, one being a lipoprotein while the other was not. From diffusion chamber studies it appears that the inhibitors or chalones were specific for CFU-c and have no affect on CFU-s. Furthermore, granulocytic chalones are produced by mature granulocytes and inhibit only granulocytic precursors.

It appears that positive and negative feedback loops regulate CFU-c. CSA as well as granulocyte chalone are candidate regulators of the committed granulocyte colony forming cells. Possibilities of interactions are discussed.